

## Diagnostic and Preventive Practices for Viral Nervous Necrosis (VNN)

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### ABSTRACT

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodaviruses (=betanodaviruses) has spread worldwide in the past decade among cultured marine fish. The present paper briefly describes procedures currently practiced in the diagnosis and prevention of the disease.

### INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodaviruses has spread worldwide, *i.e.* in Indo-Pacific region, Mediterranean, Great Britain, Scandinavia, and North America, among cultured marine fish with the number of susceptible host species continuing to grow (Munday and Nakai 1997; Curtis *et al.*, 2001; Starkey *et al.*, 2000; Grotmol *et al.*, 1997). To date, the disease has been reported in at least 25 fish species, with the greatest impact being in sea bass *Lates calcarifer* (Glazebrook *et al.*, 1990) and *Dicentrarchus labrax* (Breuil *et al.*, 1991), groupers *Epinephelus akaara* (Mori *et al.*, 1991), *E. fuscogutatus* (Chi *et al.*, 1997), *E. malabaricus* (Danayadol *et al.*, 1995), *E. moara* (Nakai *et al.*, 1994), *E. septemfasciatus* (Fukuda *et al.*, 1996), *E. tauvina* (Chua *et al.*, 1995), *E. coioides* (Lin *et al.*, 2001) and *Cromileptes altivelis* (Zafran *et al.*, 2000), striped jack *Pseudocaranx dentex* (Mori *et al.*, 1992), parrotfish *Oplegnathus fasciatus* (Yoshikoshi and Inoue, 1990), puffer *Takifugu rubripes* (Nakai *et al.*, 1994), and flatfish *Verasper moseri* (Watanabe *et al.*, 1999), *Hippoglossus hippoglossus* (Grotmol *et al.*, 1997; Starkey *et al.*, 2000), *Paralichthys olivaceus* (Nguyen *et al.*, 1994), and *Scophthalmus maximus* (Bloch *et al.*, 1991).

The causative agent was first characterized in striped jack *P. dentex* and then in barramundi *L. calcarifer*, European sea bass *D. labrax*, and grouper *Epinephelus* sp. with VNN. (Mori *et al.*, 1992; Comps *et al.*, 1994; Chi *et al.*, 2001). It is currently placed in the genus Betanodavirus, the family Nodaviridae (van Regenmortel *et al.*, 2000). Immunological studies have shown relationships between striped jack nervous necrosis virus (SJNNV, the type species of the genus Betanodavirus) and the other betanodaviruses (Munday *et al.*, 1994; Totland *et al.*, 1999; Skliris *et al.*, 2001). The genomic classification of betanodaviruses, based on partial nucleotide sequences of the coat protein gene (RMA2), has also shown that major genotypes are the SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and red spotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa *et al.*,

1997). The complete nucleotide sequences of RNA1 and RNA2 (RNA-dependent RNA polymerase) of SJNNV or GGNNV (a grouper betanodavirus) have been reported (Iwamoto *et al.*, 2001; Tan *et al.*, 2001).

In the past decade, information on the disease and betanodaviruses has accumulated and there are now many procedures available for diagnosis of the disease. The present paper briefly describes the diagnostic methods of VNN and the procedures to prevent the disease, although the prevention method has not been fully developed because of unknown infection mechanisms of the disease, particularly in grouper's VNN.

## DIAGNOSTIC METHODS

### 1. *Clinical signs and histopathology*

There are considerable variations in age at which the disease is first noted and the period over which mortality occurs. In general, the earlier the signs of disease occur, the greater is the rate of mortality. Although disease occurrence at the juvenile stage in some species is very rare, high mortalities often occur at juvenile to young stages in other fish species, but mortality usually does not reach 100%, indicating age-dependent susceptibility (OIE, 2000). Mortalities have been reported in production-sized European sea bass (Le Breton *et al.*, 1997) and sevenband grouper *E. septemfasciatus* (Fukuda *et al.*, 1996).

All diseases are characterized by a variety of neurological abnormalities such as erratic swimming behavior and vacuolation of the central nervous tissues (brain, spinal cord) and the retina. In general, younger fish have more severe lesions; older fish have less extensive lesions and these may show a predilection for the retina (Munday and Nakai, 1997). Endocarditis has been described in Atlantic halibut (Grotmol *et al.*, 1997) and neuronal necrosis has been described in most species.

Presumptive diagnosis of VNN can be made on the basis of a conspicuous vacuolation in the brain, spinal cord and/or retina. However, VNN in fish with only a few vacuoles in the nervous tissues maybe difficult to diagnose.

### 2. *Electron microscopy*

The virus particles can be visualized in affected brain, spinal cord, and retina. The virus is mainly associated with vacuolated cells and, characteristically, some inclusions. The reported particles vary in size from 22 nm (Breuil *et al.*, 1991) to 34 nm (Yoshikoshi and Inoue, 1990) arranged intracytoplasmically in crystalline arrays, or as aggregates and single particles intra- and extracellularly. The virus is nonenveloped and icosahedral in shape.

### 3. Immunological methods

All betanodaviruses can be detected either by indirect fluorescent antibody test (FAT) or immunohistochemistry (IHC) with a rabbit anti-SJNNV serum (Munday and Nakai, 1997; Munday *et al.*, 1994; Totland *et al.*, 1999). These are routinely used for confirmative diagnosis of the disease. Although other immunological methods, e.g. the enzyme-linked immunosorbent assay (ELISA) (Arimoto *et al.*, 1992; Huang *et al.*, 2001) or neutralization test (Skloris *et al.*, 2001), are available for virus identification, they may be used only for some betanodaviruses due to limited information on the serological properties of the virus.

There have been reports on the sero-diagnosis of VNN or VER using ELISA methods (Mushiake *et al.*, 1992; Mushiake *et al.*, 1993; Breuil and Romestand, 1999; Breuil *et al.*, 2000). However, due to insufficient knowledge on the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not gained acceptance as a routine screening method for assessing the viral status of fish populations.

### 4. Molecular methods

Reverse transcription-polymerase chain reaction (RT-PCR) is the most powerful tool to detect the virus not only from diseased fish but also from asymptomatic carriers. A single primer set designed to amplify the T4 region (427 bases) of SJNNV coat protein gene (Nishizawa *et al.*, 1994) is available for all genotypic variants of betanodaviruses, with only one exception (Thiery *et al.*, 1999). Although RT-PCR is convenient for the diagnosis of VNN or VER, this technique requires  $10^{4-5}$  TCID<sub>50</sub> for detection of the betanodaviruses. The sensitivity of the diagnosis is improved by nested RT-PCR (Thiery *et al.*, 1999; Della Valle *et al.*, 2000). Comps *et al.* (1996) reported *in situ* hybridization using DIG-labelled probes to examine the tissue-specific expression of betanodaviruses.

### 5. Culture in cells

All genotypic variants of betanodaviruses can be cultured in the fish cell line SSN-1 which was derived from striped snakehead *Channa striatus* (Frerichs *et al.*, 1991, Frerichs *et al.*, 1996; Iwamoto *et al.*, 1999), and a clonal cell line E-11 from the SSN-1 cells is useful for qualitative and quantitative analyses of all the betanodaviruses (Iwamoto *et al.*, 2000). It is notable that both SSN-1 and E-11 cells are infected by a spontaneously productive C-type retrovirus designated as SnRV (Frerichs *et al.*, 1991; Hart *et al.*, 1996; Iwamoto *et al.*, 2000). Virus titration and growth experiments using the E-11 cell line clearly revealed differences in the optimal growth temperature among the genotypic variants: 25 to 30°C for RGNNV genotype, 20 to 25°C for SJNNV genotype, 20°C for TPNNV, and 15 to 20°C for BFNNV. This culture system is also useful for detection of virus-neutralizing antibodies in fish serum (Tanaka *et al.*, 2001; Yuasa *et al.*, 2002). Needless to say, the culture system has opened a gate for molecular analysis of betanodaviruses (Iwamoto *et al.*, 2001).

The E-11 cells are highly sensitive to betanodaviruses but it takes a maximum of 10 days after inoculation to detect the virus at lower numbers based on the CPE expression. In contrast, the RT-PCR is generally a rapid method to examine a large number of samples but its sensitivity is not so high, as mentioned above. Accordingly, the combined procedure of cell-culture and RT-PCR techniques will be a rapid and convenient method to detect infective viral particles from asymptomatic carriers or samples with low virus levels. For example, when the 72-h culture of virus in the E-11 cells was examined by the RT-PCR, a positive-PCR amplicon was obtained from every genetic variant sample containing virus particles at the lowest number ( $10^{\circ}$  TCID<sub>50</sub>). The cultivation for 24 to 48 h prior to RT-PCR was enough to detect the virus at the lowest titer (Iwamoto *et al.*, 2001). The validity of this procedure, *i.e.* preculture in the E-11 cells and RT-PCR, has been demonstrated in the detection of a RGNNV- genotype betanodavirus from white seabass *Atractoscion nobilis* (Curtis *et al.*, 2001).

## PREVENTIVE METHODS

### 1. Prevention of vertical transmission

In VNN of striped jack, virus-carrying broodstock were shown to be the most important inoculum source of the virus to their larvae (Arimoto *et al.*, 1992; Mushiake *et al.*, 1994). This finding led to successful control of VNN in larval striped jack. Elimination or segregation of virus-carrying spawners, based on RT-PCR or cell-culture procedure just prior to spawning, is highly effective to prevent the disease, where disinfection of fertilized eggs with ozone is usually practiced for extra safety (Mushiake *et al.*, 1994; Mori *et al.*, 1998). However, the transmission mode of causative viruses in fish other than striped jack remains unclear, although betanodaviruses have been demonstrated in the gonad materials of broodstock of groupers and European sea bass (Nakai *et al.*, 1994; Comps *et al.*, 1996).

### 2. Prevention of horizontal transmission

The mode of transmission/introduction of the viruses, other than in gametes, has not been demonstrated, but the possibilities include influent water, juvenile fish held on the same site, and carriage on utensils, vehicles, etc. As betanodaviruses are quite resistant to environmental conditions (Frerichs *et al.*, 2000), it is possible that they are readily translocated by commercial activities. Horizontal transmission and introduction of the viruses via contaminated rearing water and utensils will be possible.

Anderson *et al.* (1993) reported that a regime of non-recycling of water, chemical sterilization of influent seawater and decontaminating half of the tanks during each hatching cycle was successful in preventing VNN in an Australian barramundi hatchery. In the control of VNN in larval striped jack, Arimoto *et al.* (1996) recommended the following measures: (1) disinfection of eggs with iodine or ozone and utensils with chlorine; (2) rearing of each batch of larvae/juveniles in separate tanks supplied with seawater sterilized by UV or ozone; and (3) rigorous separation of larvae/juveniles from broodfish.

### 3. Vaccination

Virus-neutralizing antibodies have been found in the serum of sevenband grouper that survived intramuscular injection with the betanodavirus, indicating establishment of acquired immunity in survivors. As many grouper species are susceptible to the virus even at the grow-out stage, vaccination is an alternative to control the disease. Intramuscular injection of the *Escherichia coli*-expressed recombinant virus coat protein, which was constructed from RGNNV genotype strains, induced virus-neutralizing antibodies in groupers and protection against challenge by homologous or heterologous RGNNV virus (Tanaka *et al.*, 2001; Yuasa *et al.*, 2002). Since this antibody-reaction is genotype-specific, a multivalent vaccine will be required for overall protection from infection by the different genotypic variants responsible for causing VNN. A similar vaccine-efficacy experiment was performed on juvenile turbot *S. maximus*, resulting in significant protection against the virus challenge (Husgaro *et al.*, 2001). The authors also showed that intraperitoneal injection of an oil-emulsified recombinant protein from SJNNV induced a specific humoral immune response in both turbot and Atlantic halibut *H. hippoglossus*. This suggests a potential for the control of VNN through maternal immunity of spawners that received the recombinant vaccine.

### 4. Reducing stress factors

In spite of the above-mentioned procedures, ultimate prophylaxis must be to reduce various stress factors happening under culture conditions. Anderson *et al.* (1993) found that reducing the stocking density of barramundi to 10 larvae/l or lower in green ponds can reduce transmission and disease to a negligible level. In the infection experiment with redspotted grouper *E. akaara*, Tanaka *et al.* (1998) reported that rearing water temperature (16-28°C) influences development of VNN: higher mortality and earlier appearance of the disease signs are observed at higher water temperatures, suggesting that manipulation of water temperature will be efficacious in reducing the disease outbreaks. Mushiake *et al.* (1994) recommended that provisionally virus-free fish (striped jack) are not induced to spawn more than 10 times in a season, because the stress of multiple spawning activates residual, extraovarian virus. Recently, a very strange phenomenon was found in hatchery-produced juvenile kelp grouper *E. moara* (Banu and Nakai, unpublished data). When apparently healthy fish in a pond were examined by RT-PCR, nodavirus was detected from the brains at high frequency. In order to follow-up this phenomenon, virus-free kelp grouper were injected intramuscularly with a betanodavirus. As a result, the inoculated virus was isolated from the brains and eyes at very high titers, the maximum  $10^9$  TCID<sub>50</sub>/g, but no disease signs were noticed in fish. Probably, unknown stress factors involved in rearing process of fish induce this inapparent infection to apparent infection. Therefore, it is essential to identify such stress factors in rearing of fish.

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